Multiplex Serum Biomarker Assays Improve Prediction of Renal and Mortality Outcomes in Chronic Kidney Disease.

William P. Martin¹, Chloe Conroy², Serika D. Naicker³, Sarah Cormican³,⁴, Tomás P. Griffin³,⁵, Md Nahidul Islam³, Eibhlin M. McCole⁶, Ivan McConnell⁷, John Lamont⁷, Peter FitzGerald⁷, John P. Ferguson⁸, Ciarán Richardson⁶, Susan E. Logue³, Matthew D. Griffin³,⁴

¹Diabetes Complications Research Centre, School of Medicine, Conway Institute, University College Dublin, Ireland.
²School of Medicine, University College Dublin, Ireland.
³Regenerative Medicine Institute (REMEDi) at CÚRAM Centre for Research in Medical Devices, School of Medicine, National University of Ireland, Galway, Ireland.
⁴Nephrology Services, Galway University Hospitals, Saolta University Health Care Group, Galway, Ireland.
⁵Centre for Diabetes, Endocrinology and Metabolism, Galway University Hospitals, Saolta University Health Care Group, Galway, Ireland.
⁶Randox Teoranta, Meenmore, Dungloe, Co. Donegal.
⁷Randox Laboratories Limited, 55 Diamond Road, Crumlin, Co. Antrim.
⁸HRB Clinical Research Facility, Galway, Ireland.

Correspondence to:
Prof. Matthew Griffin,
Regenerative Medicine Institute (REMEDi),
CÚRAM Centre for Research in Medical Devices,
School of Medicine,
College of Medicine, Nursing and Health Sciences,
National University of Ireland, Galway,
Ireland.
+353-91-495436
matthew.griffin@nuigalway.ie

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Key Points

- Incorporation of 11 serum biomarkers alongside clinical variables improved prediction of adverse CKD outcomes over 5-year follow-up.
- Patients with the triad of high sTNFR1 and NGAL coupled with low C3a-desArg had particularly high adverse event rates during follow-up.
- Biomarkers were quantified on a single, clinical-grade analyser, with potential for improved translatability to the CKD outpatient setting.

Abstract

Background: We investigated the predictive value of 11 serum biomarkers for renal and mortality endpoints in people with chronic kidney disease (CKD).

Methods: Adults with CKD (n=139) were enrolled from outpatient clinics between February 2014 and November 2016. Biomarker quantification was performed using two multiplex arrays on a clinical-grade analyser. Relationships between biomarkers and renal and mortality endpoints were investigated by random forests and Cox proportional hazards regression.

Results: The cohort was 56% male. Mean age was 63 years and median [IQR] CKD-EPI eGFR was 33 [24-51] mL/min/BSA. Fifty-six (40%) people developed a composite endpoint defined as ≥40% decline in eGFR, doubling of serum creatinine, renal replacement therapy, or death over median follow-up of 5.4 [4.7-5.7] years. Prediction of the composite endpoint was better with random forests trained on serum biomarkers compared with clinical variables (area under the curve 0.81 vs 0.78). Predictive performance of biomarkers was further enhanced when considered alongside clinical variables (area under the curve 0.83 vs 0.81
for biomarkers alone). Patients (n=27, 19%) with high soluble tumour necrosis factor receptor-1 (≥3 ng/mL) and neutrophil gelatinase-associated lipocalin (≥156 ng/mL) coupled with low complement 3a des-arginine (<2,368 ng/mL) almost universally (96%) developed the composite renal and mortality endpoint. C-reactive protein (adjusted hazard ratio, 1.4; 95% confidence interval, 1.1 to 1.8), neutrophil gelatinase-associated lipocalin (adjusted hazard ratio, 2.8; 95% confidence interval, 1.3 to 6.1) and complement 3a des-arginine (adjusted hazard ratio, 0.6; 95% confidence interval, 0.4 to 0.96) independently predicted time to the composite endpoint.

Conclusions: Outpatients with the triad of high soluble tumour necrosis factor receptor-1 and neutrophil gelatinase-associated lipocalin coupled with low complement 3a des-arginine had high adverse event rates over 5-year follow-up. Incorporation of serum biomarkers alongside clinical variables improved prediction of CKD progression and mortality. Our findings require confirmation in larger, more diverse patient cohorts.
Introduction

Chronic kidney disease (CKD) is a growing public health problem, with its prevalence increasing by 29.3% since 1990 to affect 9.1% of the global population in 2017 (1). Although morbidity and mortality rates from other non-communicable diseases have declined over the past 3 decades, no such favourable trends exist for CKD (2). Death due to cardiovascular disease is over-represented amongst people with CKD and mortality rates increase as estimated glomerular filtration rate (eGFR) declines. In a meta-analysis of 21 general population cohorts incorporating over 1.2 million participants, eGFR independently predicted mortality risk in an almost linear fashion (3).

Communicating risk of adverse outcomes to patients with CKD is challenging, particularly at earlier, typically asymptomatic disease stages. Although eGFR and urine albumin-to-creatinine ratio (uACR) are strongly predictive of adverse outcomes in epidemiological studies, intra-individual variability weakens their prognostic value in clinical practice (4, 5). Measuring multiple circulating biomarkers simultaneously has the potential to uncover subgroups of patients with CKD who have differing risks of progressive renal functional decline and mortality. However, prognostication of adverse CKD outcomes with multiple biomarkers is challenged by the strong inter-correlation between biomarkers from diverse pathways, which may result in marginal improvements in predictive performance when additional biomarkers are studied (6-8). Additionally, many biomarker studies to date have enrolled specific subgroups of patients with CKD, for example diabetic kidney disease; predictive performance of circulating biomarkers across the spectrum of CKD severity and aetiology in real-world outpatient nephrology practice is underexplored.

We aimed to evaluate the performance of two multi-analyte serum biomarker arrays in
patients with CKD. Specifically, we aimed to ascertain the relationships of the 11 biomarkers to each other and to determine their individual and combined predictive value for renal and mortality outcomes. We hypothesised that clusters of patients with differing risks of adverse CKD outcomes could be identified based on serum biomarker profiles. Furthermore, we hypothesised that incorporation of multiple biomarkers into multivariate models would improve prediction of renal and mortality endpoints over 5-year follow-up compared with clinical variables alone in people with a broad range of CKD aetiologies and severity attending a tertiary referral nephrology centre.

**Materials and Methods**

**Study Cohort**

Adults with CKD stages 1 to 5 were enrolled from nephrology outpatient clinics at Galway University Hospitals between February 2014 and November 2016. As previously described (9), inclusion criteria were: age ≥18 years, diagnosis of CKD, absence of current infection, immunosuppression, cancer, acute cardiovascular event or haematological condition other than anaemia, haemoglobin ≥10 g/dL, not on renal replacement therapy (RRT), and no prior kidney transplant.

Clinical and laboratory data were recorded from enrolment to the end of follow-up on July 15th, 2020 in a secure, password-protected, web-based clinical database (Distiller®, SlidePath, Ireland). Longitudinal measurements of serum creatinine were extracted for each participant using the eMEDRenal® system (Mediqal H.I., Aston, UK). An IDMS-traceable creatininase assay was used to measure creatinine (10). CKD-EPI eGFR was calculated from serum creatinine using standard formulae and expressed as mL/min/body surface area.
(BSA). Second and subsequent creatinine values on a given day and creatinine values
subsequent to RRT initiation were excluded. Individuals with <3 eGFR values were excluded
from the dataset. Interim renal outcomes (≥40% decline in Chronic Kidney Disease-
Epidemiology Collaboration [CKD-EPI] eGFR and doubling of serum creatinine) were defined
at their first occurrence after study enrolment compared with baseline study values. RRT
was defined as first requirement for dialysis or kidney transplantation after study
enrolment. All-cause mortality after study enrolment was recorded. Time to event of renal
and mortality endpoints was recorded for each patient. Duration of study follow-up was
calculated as time from enrolment to date of last study follow-up or death. Duration of
renal functional follow-up was calculated as time from enrolment to date of last eGFR
determination. Annual eGFR slopes were calculated by linear regression of eGFR over time
for individuals with ≥3 eGFR determinations over ≥1 year. Median log-transformed uACR
was estimated from uPCR using the validated equation of Weaver et al. (11). Four-variable
kidney failure risk equation (KFRE) scores for 2- and 5-year risks of progression to kidney
failure were calculated according to regional (non-North American) formulae (12).

The study was approved by the Galway University Hospitals Clinical Research Ethics
Committee (reference C.A. 885) and conducted in accordance with the 1964 Helsinki
declaration. Participants provided written, informed consent.

Blood Sample Collection and Serum Biomarker Determinations

Serum was isolated from peripheral venous blood samples provided at enrolment.

Eleven serum biomarkers were quantified using two CKD multiplex arrays on an Evidence
Investigator® immunoassay analyser (Randox Teoranta, Donegal, Ireland). As physiological
serum concentrations vary significantly for the 11 included biomarkers (from pg/mL to
μg/mL), lowly and highly abundant biomarkers were quantified on separate multiplex arrays to improve assay sensitivity and performance. A 7-analyte array measured low-abundance biomarkers: epidermal growth factor (EGF), interleukin-8, soluble tumour necrosis factor receptor-1 (sTNFR1) and receptor-2 (sTNFR2), fatty acid-binding protein-1 (FABP1), D-dimer, and macrophage inflammatory protein-1-alpha (MIP-1-alpha); a 4-analyte array measured highly abundant biomarkers: C-reactive protein, cystatin C, C3a with cleaved C-terminal arginine (C3a-desArg), and neutrophil gelatinase-associated lipocalin (NGAL). Additional details on serum isolation, the immunoassay procedure, the rationale behind biomarker selection, and assay performance and validation are provided in the Supplemental Material.

Inter-assay coefficient of variation for individual biomarkers across multiplex array plate runs used to quantify biomarkers for the study cohort is presented in Supplemental Table 1. Inter-assay coefficient of variation was 8.0% for the 7-analyte array and 10.3% for the 4-analyte array, resulting in an overall inter-assay coefficient of variation of 9.2%.

Statistical Analyses

Descriptive and Inferential Statistics, Logistic Regression, and Clustering

RStudio® version 4.0.0 was used for analysis. A composite renal and mortality endpoint of ≥40% decline in eGFR, doubling of serum creatinine, RRT, or death was defined and used as the primary outcome for analyses. A renal-specific composite endpoint of ≥40% decline in eGFR, doubling of serum creatinine, or RRT was used in sensitivity analyses. Biomarkers were transformed using the natural logarithm for analysis. Cohort characteristics were summarised by descriptive statistics. Independent sample t-tests, Wilcoxon rank-sum tests, and χ² tests assessed for differences in clinical variables and biomarkers amongst those who did and did not develop the composite renal and mortality endpoint. P <0.05 was
considered statistically significant. Univariate relationships between serum biomarkers and the composite renal and mortality endpoint were investigated using logistic regression. Odds ratios from logistic regression models are expressed per one unit change in natural logarithm biomarker concentrations. Unsupervised clustering of patients based on biomarker concentrations was performed by principal components analysis (13).

**Decision Tree and Random Forest Classification Models**

A supervised machine learning approach with binary classification random forests was used to explore the value of all biomarkers considered together in predicting the composite renal and mortality endpoint. Random forests have several advantages over logistic regression, including seamless handling of regressor collinearity and automatic selection and fitting of non-linear relationships and statistical interactions. A binary classification decision tree evaluating classification of the composite renal and mortality endpoint by serum biomarkers was generated using default parameters of the R function ‘rpart’ to illustrate the complementary information provided by multiple biomarkers (14, 15). Biomarkers were inputted to the decision tree and random forest models as continuous variables. Biomarker thresholds were selected by recursive binary splitting to maximise node purity in each tree (the number of individuals from a single class, who either did or did not develop the composite renal and mortality endpoint). Binary classification random forests (5,000 trees per model), with the composite renal and mortality endpoint as the response variable, were fit (16). Three model types were created: clinical variables alone (age, gender, hypertension, diabetes, and eGFR), biomarkers alone, and clinical variables plus biomarkers. A leave-one-out cross-validation approach was implemented, which consisted of excluding one individual in turn from training the random forest model. Subsequently, the trained random
forest model predicted the class of the individual excluded during model training. This process was repeated iteratively for each individual in the dataset such that a predicted class was assigned to each study participant by each of the three random forest model types. The leave-one-out cross-validation procedure was only performed for random forest models and not for other model types.

Area under the curve (AUC) values were calculated for each of the three model types using the predicted probability of having developed the composite renal and mortality endpoint and the individual’s actual recorded composite renal and mortality endpoint status as inputs to the function ‘roc’ in the R package pROC (17). Receiver operating characteristic curves were plotted using the function ‘ggroc’. Model performance metrics (sensitivity, specificity, positive predictive value, and negative predictive value) were calculated across three probability thresholds (10%, 30%, and 50%) for labelling patients as having developed the composite renal and mortality endpoint. For example, an individual with a predicted probability of the composite renal and mortality endpoint by a random forest model of 45% would be labelled as having developed the composite renal and mortality endpoint by the first two thresholds evaluated, but not the latter. An estimate of variable importance to the random forest models (mean decrease in accuracy) was calculated from mean values of each model iteration during the leave-one-out cross-validation procedure.

Cox Proportional Hazards Regression Models

Multivariable Cox proportional hazards regression models were created to investigate relationships between biomarkers and time to renal and mortality and renal-specific composite endpoints. The primary Cox model analysis investigated the value of biomarkers when considered in addition to clinical variables in predicting time to the composite renal and mortality endpoint. For this analysis, two models were constructed: a clinical model
adjusting for age, gender, hypertension, diabetes, and eGFR; and a clinical plus biomarker model additionally incorporating all log-transformed biomarker values. Backward elimination of non-significant biomarker effects from the clinical plus biomarker model was subsequently performed using stepAIC (18). Biomarkers for which the p-values for their hazard ratios were greater than 0.05 were manually excluded from the Akaike Information Criterion (AIC)-selected model to create a final parsimonious clinical plus biomarker model. Clinical variables were manually retained in the final model. Hazard ratios from Cox models are expressed per one unit change in natural logarithm biomarker concentrations. Comparisons of Cox model adequacy (clinical model versus clinical plus biomarker model) were assessed using likelihood ratio $\chi^2$ tests.

Cox models were constructed using the R package survival (19). The R package survminer was used to create Forest plots and to plot fully adjusted survival curves from the multivariate clinical plus biomarker Cox model for the composite renal and mortality endpoint according to biomarker tertiles, which were calculated using the function ‘ntile’ from the R package dplyr (20, 21). We tested each Cox model for proportionality assumptions using Schoenfeld residuals. AIC values for fitted Cox models were obtained using extractAIC (22).

**Sensitivity Analyses**

A series of sensitivity analyses was performed to further evaluate the predictive value of serum biomarkers incorporated in random forest and Cox models outlined above. Random forest models trained on clinical variables, alone or in combination with biomarkers, were additionally adjusted for baseline uACR. Baseline uACR was included in random forest models in two separate analyses: firstly, random forest models were performed in the
subgroup with baseline uACR data available and secondly, after imputation of missing baseline uACR data using the function ‘rffImpute’ in the R package randomForest (16). Imputation of missing uACR data was only performed for sensitivity analyses involving random forests.

With respect to the final parsimonious clinical plus biomarker Cox model of the composite renal and mortality endpoint outlined above, the predictive value of biomarkers in this model was separately evaluated in Cox models in the subgroup with CKD stages 3-5/eGFR <60 mL/min/BSA and in the subgroup with baseline uACR data available. The value of these biomarkers for predicting time to a renal-specific composite endpoint was also evaluated using Cox models, both in the full study cohort and in the subgroup with baseline uACR data available.

Results

Baseline Characteristics and Serum Biomarker Concentrations

Baseline characteristics and serum biomarker concentrations of the study cohort (n=139) stratified by development (n=56) or not (n=83) of the composite renal and mortality endpoint are presented in Table 1. The study population had a mean age of 63 years, 56% were male, and median [IQR] eGFR was 33 [24 - 51] mL/min/BSA. Of those sampled, study participants had moderate proteinuria with a median [IQR] uACR of 127 [22 - 479] mg/g and uPCR of 327 [124 - 814] mg/g. After calculating uACR from uPCR (11), baseline uACR data were available for 113 (81%) individuals for whom the median [IQR] uACR was 144 [22 - 532] mg/g. Characteristics of individuals with available and missing baseline uACR data (after conversion of uPCR to uACR where available) are presented in Supplemental Table 2. Those with missing uACR data trended to be older (68±14 vs 62±17 years, p=0.06);
otherwise no significant differences between those with and without baseline uACR data were observed. Over 25% and 80% of the study cohort had diabetes mellitus and hypertension, respectively, while glomerulonephritis (22%) and diabetic kidney disease (17%) were the two most documented CKD aetiologies. Median [IQR] 2- and 5-year risks of progression to kidney failure were 1.4 [0.3 – 6.5] and 5.4 [1.2 – 23.1] % respectively.

Compared with individuals who did not develop the composite renal and mortality endpoint, those who did were older (67±15 vs 60±17 years, p=0.01), more likely to be male (71% vs 46%, p=0.005), had a higher prevalence of diabetes mellitus (43% vs 13%, p<0.001), lower eGFR (26 [18 - 34] vs 43 [31 - 60] mL/min/BSA, p<0.001), higher uACR (283 [83 - 993] vs 104 [19 - 269] mg/g, p<0.001), and higher 5-year KFRE scores (22.5 [6.1 – 55.1] vs 2.1 [0.3 – 9.0] %, p<0.001). Concentrations of several serum biomarkers were higher in those who developed the composite renal and mortality endpoint, including sTNFR1, sTNFR2, NGAL, cystatin C, and to a lesser extent C-reactive protein, FABP1, and MIP-1-alpha. Conversely, concentrations of EGF and C3a-desArg were lower in those who developed the composite renal and mortality endpoint. Of note, the median sTNFR2 concentration (1.4 [0.8 – 2.2] ng/mL) was lower than that of sTNFR1 (3.0 [2.1 – 4.6] ng/mL) in the study cohort, which is the opposite to what has been observed in several studies (23-25). However, sTNFR1 and sTNFR2 concentrations remained strongly correlated with each other (Pearson r correlation 0.73, p<0.001) (Supplemental Figure 1).

**Incidence of Renal and Mortality Endpoints**

Median [IQR] duration of study follow-up was 5.4 [4.7 – 5.7] years. Median duration of renal functional follow-up (study enrolment to date of final eGFR determination) was 4.7 [3.5 – 5.2] years, with participants having a median of 22 [12 - 34] eGFR values (Table 2). The
median rate of decline in eGFR was -0.9 [-2.3 – 0.5] mL/min/BSA/year. Renal functional decline was greater in those who developed the composite renal and mortality endpoint compared with those who did not (-2.2 [-3.9 - -1.4] vs 0 [-1 – 1.6] mL/min/BSA/year, p<0.001). Relevant study endpoints including ≥40% decline in CKD-EPI eGFR, doubling of serum creatinine, RRT, and death occurred in 38 (68%), 14 (25%), 21 (38%), and 15 (27%), respectively. Fifty-six (40%) individuals developed the composite renal and mortality endpoint while 47 (34%) developed the renal-specific composite endpoint.

Univariate Relationships Between Serum Biomarkers and the Composite Endpoint

Figure 1 presents biomarkers with significant associations with the composite renal and mortality endpoint by univariate logistic regression. EGF (odds ratio [OR], 0.5; 95% confidence interval [95% CI], 0.3 to 0.9) and C3a-desArg (OR, 0.6; 95% CI, 0.4 to 0.96) were inversely associated with, while MIP-1-alpha (OR, 1.8; 95% CI, 1.2 to 2.8) and C-reactive protein (OR, 1.6; 95% CI, 1.2 to 2.2) were positively associated with, the composite renal and mortality outcome. Furthermore, sTNFR1 (OR, 25.9; 95% CI, 8.6 to 94.5), sTNFR2 (OR, 13.7; 95% CI, 4.5 to 47.4), NGAL (OR, 4.8; 95% CI, 2.4 to 10.6), and cystatin C (OR, 10.4; 95% CI, 4.0 to 31.5) were strongly associated with the composite renal and mortality endpoint.

Clustering of Patients Based on Serum Biomarkers

Unsupervised clustering of patients by principal components analysis identified shifts in biomarkers by CKD stage and by the composite renal and mortality endpoint (Figure 2, panels A and B), with the loading plot (Figure 2, panel C) illustrating which biomarkers were important in this regard. Expression of several biomarkers clustered together in a predictable fashion based on a priori knowledge. sTNFR1 and sTNFR2 clustered together, as did two biomarkers which were inversely associated with the composite renal and mortality
endpoint: EGF and C3a-desArg. Patients separated based on biomarker expression along principal components 1 and 2 by both CKD stage and by development of the composite renal and mortality endpoint. Biomarkers clustered in the upper left corner (EGF and C3a-desArg) were more strongly expressed in earlier stage CKD and in those who did not develop the composite renal and mortality endpoint (Figure 2, panel C and Table 1). Conversely, biomarkers in the far right (NGAL, cystatin C, sTNFR1, and sTNFR2) were more strongly expressed in advanced CKD and in those who developed the composite renal and mortality endpoint (Figure 2, panel C and Table 1).

**Prediction of a Renal and Mortality Composite Endpoint by Serum Biomarkers using Supervised Machine Learning (Random Forest Classification Models)**

C3a-desArg reclassified risk of the composite renal and mortality endpoint amongst those with high sTNFR1 and NGAL (Figure 3, panel A). Those with high sTNFR1 (≥3 ng/mL), high NGAL (≥156 ng/mL), but also high C3a-desArg (≥2,368 ng/mL) had a 44% risk of the composite renal and mortality endpoint. Conversely, individuals with high sTNFR1 (≥3 ng/mL), high NGAL (≥156 ng/mL), and low C3a-desArg (<2,368 ng/mL), which accounted for 19% of the cohort, almost universally (96%) developed the composite renal and mortality endpoint.

Receiver operating characteristic (ROC) curves and AUC values for classification of the composite renal and mortality endpoint by random forests trained on clinical variables alone, biomarkers alone, and clinical variables plus biomarkers are presented in Figure 3, panel B. An incremental improvement in predictive performance was observed between models trained on biomarkers compared with those trained on clinical variables (AUC 0.81 vs 0.78). Predictive performance was further enhanced after inclusion of clinical variables.
alongside biomarkers to train the models (AUC 0.83 vs 0.81 for biomarkers alone). Baseline eGFR was the most important predictor of the composite renal and mortality endpoint in models trained on clinical variables alone, while, when incorporated alongside biomarkers, eGFR and cystatin C were ranked as the third and fourth most important variables, respectively (Figure 3, panels C-E). sTNFR1 and NGAL were the 2 most important variables to prediction of the composite renal and mortality endpoint, both when biomarkers were considered alone and alongside clinical variables.

Additional random forest performance metrics stratified by three predicted probability thresholds (10%, 30%, and 50%) for classifying the composite renal and mortality endpoint are presented in Table 3. At a low (10%) predicted probability threshold, inclusion of serum biomarkers improved sensitivity for classification of the composite endpoint at the expense of reduced specificity. At the 30% predicted probability threshold, models trained on serum biomarkers again improved sensitivity for classification of the composite endpoint while also achieving specificity values comparable to models trained on clinical variables alone. At both of these thresholds, highest sensitivity for classification of the composite endpoint was observed when serum biomarkers were considered alongside clinical variables. At the 50% predicted probability threshold, favourable trends towards improved sensitivity and specificity were observed in models trained on serum biomarkers, albeit the absolute magnitude of improvement was smaller than at lower predicted probability thresholds.

Sensitivity analyses were performed to test the extent to which improved classification of the composite renal and mortality endpoint by random forest models incorporating serum biomarkers persisted after inclusion of uACR alongside other clinical variables. While AUC values of random forest models trained on clinical variables alone did improve after
incorporation of baseline uACR using two different approaches, the highest AUC values continued to be observed in random forest models trained on both clinical variables plus serum biomarkers (Supplemental Figure 2, panels A-B).

Prediction of Time to Renal and Mortality and Renal-Specific Composite Endpoints by Serum Biomarkers (Cox Models)

The AIC value for the Cox model incorporating all clinical and biomarker variables was 382.92, which reduced to 374.83 after stepwise backward elimination of six biomarkers in the following order: epidermal growth factor, FABP1, cystatin C, interleukin-8, MIP-1-alpha, and sTNFR2. Three biomarkers were included in the final clinical plus biomarker model: C-reactive protein, NGAL, and C3a-desArg. Compared with a Cox model incorporating only clinical variables (AIC 385.31), this parsimonious clinical plus biomarker model (AIC 375.86) improved prediction of time to the composite renal and mortality endpoint (p=0.001). C-reactive protein (adjusted hazard ratio [aHR], 1.4; 95% CI, 1.1 to 1.8) and NGAL (aHR, 2.8; 95% CI, 1.3 to 6.1) were positively associated with the composite renal and mortality endpoint (Figure 4, panel A). C3a-desArg was inversely associated with the composite renal and mortality endpoint (aHR, 0.6; 95% CI, 0.4 to 0.96). Figure 4, panels B-D present survival without the composite renal and mortality endpoint in the fully adjusted multivariate clinical plus biomarker Cox model stratified by tertiles of C-reactive protein (B), NGAL (C) and C3a-desArg (D).

Several sensitivity analyses were performed to further interrogate these findings. In Cox models performed in the subgroup of patients with CKD stages 3-5/eGFR <60 mL/min/BSA, serum biomarkers improved prediction of time to the composite renal and mortality endpoint (p=0.001). C-reactive protein, NGAL, and C3a-desArg remained independently
predictive of time to the composite renal and mortality endpoint, with hazard ratios similar to those obtained in the full study cohort observed (Supplemental Figure 3, panel A). In Cox models additionally adjusted for uACR, which were performed in the subgroup of patients with baseline uACR available, serum biomarkers improved prediction of time to the composite renal and mortality endpoint (p=0.001). C-reactive protein (aHR, 1.5; 95% CI, 1.1 to 2.1) and NGAL (aHR, 3.5; 95% CI, 1.5 to 8.2) remained predictive of the composite renal and mortality endpoint (Supplemental Figure 3, panel B).

Sensitivity analyses were also performed to test the predictive value of the serum biomarkers with respect to a renal-specific composite outcome, defined as ≥40% decline in CKD-EPI eGFR, doubling of serum creatinine, or RRT. Compared with a Cox model incorporating only clinical variables (AIC 329.01), the clinical plus biomarker model (AIC 325.00) improved prediction of time to the renal-specific composite endpoint (p=0.02). NGAL (aHR, 3.0; 95% CI, 1.3 to 6.7) and C3a-desArg (aHR, 0.5; 95% CI, 0.3 to 0.9) independently predicted time to the renal-specific composite endpoint (Supplemental Figure 4, panel A). In Cox models additionally adjusted for uACR, serum biomarkers that improved prediction of time to the renal-specific composite endpoint (p=0.03). NGAL (aHR, 2.7; 95% CI, 1.1 to 6.3) remained predictive of the renal-specific composite endpoint (Supplemental Figure 4, panel B).

Discussion

This study provides insight into the value of 11 serum biomarkers measured using two multiplex biochip arrays in the identification of outpatients with CKD at high-risk of accelerated renal functional decline and mortality. Biomarkers were quantified on a single, clinical-grade platform, which offers a route for improved translatability to the CKD
outpatient setting where biomarkers may enhance prognostication afforded by existing clinical risk prediction tools (12, 26). Global shifts in serum biomarker profiles, reflecting changes in both protective factors and injury markers, were evident with declining kidney function and in those who subsequently developed a composite renal and mortality endpoint defined on the basis of CKD progression, need for RRT, or death. We demonstrate, using random forests and multivariate Cox models, that incorporation of biomarkers alongside clinical variables improves prediction of CKD progression and mortality.

We employed diverse statistical methods including univariate logistic regression (for ease-of-interpretability and visual representation), principal components analysis (to illustrate clustering of patients based on serum biomarker profiles), random forest models (to accurately characterise the predictive value of the biomarkers for adverse outcomes, not least due to seamless handling of regressor collinearity), and Cox models (to incorporate time-to-event data). Replication of the overarching results (improved predictive performance of models incorporating serum biomarkers for adverse CKD outcomes) using a variety of statistical approaches adds to the robustness of the present study findings.

Annual decline in kidney function was moderate in our study cohort at -0.9 mL/min/BSA/year, which was significantly lower than a local cohort of patients with type 2 diabetic kidney disease (mean eGFR 47 mL/min/BSA) attending our hospital for multidisciplinary CKD care, in whom eGFR trajectories ranged from -6 mL/min/BSA/year pre-intervention to -3 mL/min/BSA/year post-intervention (10). However, the latter cohort of patients exclusively had type 2 diabetes of median 10 years’ duration and were enrolled up to a decade before patients in the current study (10). eGFR slope trajectory of the current cohort was more similar to, albeit still lower than, a group of patients with type 2 diabetes and CKD (mean eGFR 42 mL/min/BSA) enrolled in Dublin, Ireland during 2014-2015 in whom
annual change in eGFR was -2 mL/min/BSA/year (27). Thus, improvements in CKD management and a lower prevalence of diabetes (25%) in the present study cohort may explain the modest rates of renal functional decline observed.

Interestingly, patients with the triad of high sTNFR1 (≥3 ng/mL) and NGAL (≥156 ng/mL) coupled with low C3a-desArg (<2,368 ng/mL) almost universally (96%) developed an adverse renal outcome or died during follow-up. This finding requires validation in a larger external cohort, but does suggest that this biomarker signature may be useful for enrolment of high-risk patients with CKD into prospective studies and may also prove useful as a means of guiding treatment intensification in nephrology practice (28). The discriminant value of sTNFR1 and NGAL for adverse outcomes was improved by C3a-desArg, highlighting that proteins with diverse functions in innate immune responses and inflammation may have complementary prognostic benefit.

To the best of our knowledge, circulating C3a-desArg has not previously been explored as a biomarker of adverse CKD outcomes. C3a-desArg, also known as acylation-stimulating protein, is an adipokine that binds to the complement 5a receptor-2 to regulate metabolic processes, specifically stimulation of triglyceride accumulation and synthesis in adipocytes and glucose uptake in pancreatic β-cells (29-31). The majority of C3a-desArg is produced by the alternative complement pathway (32). However, adiponectin activates C1q, which generates C3a and C5a fragments via classical pathway activation (33). Increased plasma C3a-desArg has been documented in patients with nephrotic syndrome, and may be implicated in the pathogenesis of the associated dyslipidaemia (34, 35). In the present study, circulating C3a-desArg was inversely associated with adverse CKD outcomes. We hypothesise that decreased C3a-desArg lowers glucose uptake and decreases clearance of triglycerides and fatty acids, thereby promoting glucose intolerance, hyperinsulinaemia, and
renal glucotoxicity and lipoxicity (36). Decreased C3a-desArg may partly occur due to obesity-associated hypoadiponectinaemia (33, 36). By extension, adverse renal outcomes in those with low C3a-desArg in our cohort may be partly accounted for by loss of the protective effects of adiponectin on glomerular podocytes – if so, this may be reversed by intentional weight loss strategies (37).

Random forests trained on serum biomarkers were superior to models trained on clinical variables in terms of prognosticating CKD progression and death. Predictive performance of the biomarkers was further enhanced when incorporated alongside clinical variables. sTNFR1 and NGAL were the two most important biomarkers to classification by random forests, reaffirming their predictive value which has been demonstrated across multiple cohorts (23-25, 27, 38-40). C-reactive protein ranked as the fifth most important variable to random forest classification of the composite renal and mortality endpoint, both in models trained on serum biomarkers alone and in models trained on both clinical variables and biomarkers, which further endorses its role in prognosticating the risk of renal functional decline in CKD (41, 42). Both C-reactive protein and NGAL independently predicted time to the composite renal and mortality endpoint, which persisted after additional adjustment for baseline albuminuria and in the subgroup of patients with baseline eGFR <60 mL/min/BSA. Serum NGAL also independently predicted time to a renal-specific composite endpoint, which persisted after additional adjustment for albuminuria.

The study cohort had a high prevalence of glomerulonephritis at 22%. Indeed, glomerulonephritis was the most documented CKD aetiology in the study cohort. A significant proportion of biomarker research in CKD has focused on the subgroup of patients with DKD, although the CRIC cohort has investigated biomarker prognostication in study populations with CKD who do not exclusively have diabetes (43). Immunosuppressive
therapy in people with glomerulonephritis has the potential to modify associations between biomarkers and adverse CKD outcomes (44), however active immunosuppression was an exclusion criterion for the present study cohort. Several of the biomarkers which demonstrated the strongest independent predictive value for adverse CKD outcomes in the present study cohort have also demonstrated associations with histopathological markers of disease severity and risk of renal functional decline in biopsy-proven glomerular diseases such as IgA nephropathy and lupus nephritis, including sTNFR1, NGAL, and C-reactive protein (45-48).

Absolute sTNFR2 concentrations were lower than those of sTNFR1 in the study cohort, which is in contrast to other studies evaluating the prognostic value of both biomarkers in patients with CKD (23-25). At the time of development of the multiplex arrays, limited international reference material was available by which to standardise and harmonise the assays. More generally, assay standardisation and harmonisation remains a significant challenge in biomarker research (49). Nevertheless, the multiplex arrays used in the current study are specific for the 11 target biomarkers contained therein. Indeed, sTNFR1 and sTNFR2 concentrations were strongly correlated with each other and the two biomarkers clustered together by principal components analysis. Furthermore, sTNFR1 and sTNFR2 were the two biomarkers which were most strongly associated with development of the composite renal and mortality endpoint by univariate logistic regression, and both biomarkers ranked as important variables to correct classification of the composite renal and mortality endpoint by random forest models. Thus, the multiplex arrays are specific for both sTNFR1 and sTNFR2 targets. Although quantification of sTNFR2 by the multiplex arrays appears to provide absolute serum concentrations that are lower than those reported by
others using stand-alone immunoassays, this did not affect interpretation of the prognostic value of either sTNFR1 or sTNFR2 in the study cohort.

Our study cohort was relatively small which limited statistical power, but event rates of renal and mortality outcomes were high and findings were replicated using random forests and Cox models. Ethnicity data were missing, although the majority (>95%) of patients attending nephrology clinics in Ireland are Caucasian (10, 50). The predictive value of our multiplex arrays requires validation in a larger, more ethnically diverse cohort. Additionally, a head-to-head comparison of the value of the KFRE equation with that of the multiplex biomarker arrays for prediction of kidney failure should be performed in a subsequent, larger-scale study (12). Furthermore, prediction of incident CKD by the multiplex biomarker arrays in populations without established CKD should be evaluated and compared with existing clinical risk prediction tools such as the equations developed by the CKD Prognosis Consortium (26). Baseline albuminuria data were missing for over half of the cohort. We calculated log-transformed uACR from uPCR using a validated equation (11), providing uACR values for over 80% of participants. Individuals with missing uACR data trended to be older but there were no significant differences between those with available and missing uACR data in terms of CKD stage or aetiology, suggesting that uACR data was largely missing at random. Furthermore, we performed sensitivity analyses to demonstrate persistence of the predictive value of serum biomarkers in random forest and Cox models despite additional adjustment for albuminuria.

In summary, simultaneous measurement of 11 serum biomarkers using novel multiplex biochip array technology was technically feasible and robust. Parallel assessment of multiple biomarkers provided complementary prognostic value. In particular, patients with the triad of high sTNFR1, high NGAL, and low C3a-desArg almost universally developed an adverse
renal endpoint or died over 5-year follow-up. Incorporation of serum biomarkers alongside clinical variables improved prediction of CKD progression and mortality. Our findings provide a strong basis for focusing the content of multi-analyte biomarker panels as risk prediction tools in the CKD outpatient setting, and for confirmation of their clinical value and cost-effectiveness in larger, more diverse patient cohorts.

**Abbreviations**

95% CI: 95% confidence interval  
aHR: adjusted hazard ratio  
AIC: Akaike Information Criterion  
AUC: area under the curve  
BSA: body surface area  
C3a-desArg: complement protein 3a des-arginine (cleaved at C-terminal arginine)  
CKD: chronic kidney disease  
CKD-EPI: Chronic Kidney Disease-Epidemiology Collaboration  
EGF: epidermal growth factor  
eGFR: estimated glomerular filtration rate  
FABP1: fatty acid-binding protein-1  
IQR: interquartile range  
KFRE: kidney failure risk equation  
MIP-1-alpha: macrophage inflammatory protein-1-alpha  
NGAL: neutrophil gelatinase-associated lipocalin  
OR: odds ratio  
RRT: renal replacement therapy  
SD: standard deviation  
sTNFR1: soluble tumour necrosis factor receptor-1  
sTNFR2: soluble tumour necrosis factor receptor-2  
T1: tertile 1  
T2: tertile 2  
T3: tertile 3
uACR: urine albumin-to-creatinine ratio
uPCR: urine protein-to-creatinine ratio

Disclosures
E.M. McCole and C. Richardson are employed by Randox Teoranta and do not own any shares. I. McConnell and J. Lamont are employed by Randox Laboratories Limited and do not own any shares. J. Lamont is named as an inventor on a patent filed on the results. P. Fitzgerald reports the following: Ownership Interest: Randox Laboratories Limited. T. Griffin reports the following: Honoraria: Novonordisk, Sanofi. M. Griffin reports the following: Honoraria: American Society of Nephrology, National Institutes of Health, Hebei Medical University, China; Scientific Advisor or Membership: Editorial Boards for JASN, Kidney International, Transplantation, Frontiers in Antigen Presenting Cell Biology and Frontiers in Renal Pharmacology, Section Editor- Mayo Clinic Proceedings. All remaining authors have nothing to disclose.

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Author Contributions:
W Martin: Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Writing - original draft; Writing - review and editing
C Conroy: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review and editing
S Naicker: Data curation; Formal analysis; Investigation; Methodology; Writing - review and editing
S Cormican: Data curation; Formal analysis; Investigation; Methodology; Supervision; Writing - review and editing
T Griffin: Conceptualization; Formal analysis; Methodology; Supervision; Writing - review and editing
M N Islam: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing - review and editing
E McCole: Data curation; Formal analysis; Investigation; Methodology; Writing - review and editing
I McConnell: Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Resources; Writing - review and editing
J Lamont: Conceptualization; Formal analysis; Funding acquisition; Methodology; Resources; Writing - review and editing
P FitzGerald: Conceptualization; Formal analysis; Funding acquisition; Methodology; Writing - review and editing
Supplemental Material

Blood Sample Collection and Serum Isolation.

Immunoassay Procedure for Serum Biomarker Determinations.

Biomarker Selection, Assay Performance, and Validation.

Supplemental Table 1. Inter-Assay Coefficient of Variation of Multiplex Serum Biomarker Arrays.

Supplemental Table 2. Characteristics of Study Participants with Available and Missing Baseline uACR Data.

Supplemental Figure 1. Scatterplot of sTNFR1 and sTNFR2 reveals a strong positive correlation between both biomarkers.

Supplemental Figure 2. Sensitivity analyses of random forest classification models incorporating additional uACR adjustment illustrate added predictive value of serum biomarkers for a composite renal and mortality endpoint.

Supplemental Figure 3. Sensitivity analyses examining the predictive value of serum biomarkers with respect to time to development of a composite renal and mortality endpoint (Cox proportional hazards regression).
Supplemental Figure 4. Sensitivity analyses examining the predictive value of serum biomarkers with respect to time to development of a renal-specific composite endpoint (Cox proportional hazards regression).
References


evidence from a cross-sectional study in a tertiary nephrology centre. *Clinical obesity*: e12402, 2020 10.1111/cob.12402
Table 1. Baseline Characteristics and Serum Biomarker Concentrations of the Study Cohort Stratified by Development of a Composite Renal and Mortality Endpoint (n = 139).a,b,c

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Data available (n (%))</th>
<th>Total cohort (n = 139)</th>
<th>Did not develop composite endpoint (n = 83)</th>
<th>Developed composite endpoint (n = 56)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (mean±SD; years)</td>
<td>139 (100)</td>
<td>63±17</td>
<td>60±17</td>
<td>67±15</td>
</tr>
<tr>
<td>Male (n (%))</td>
<td>139 (100)</td>
<td>78 (56)</td>
<td>38 (46)</td>
<td>40 (71)</td>
</tr>
<tr>
<td>Diabetes mellitus (n (%))</td>
<td>139 (100)</td>
<td>35 (25)</td>
<td>11 (13)</td>
<td>24 (43)</td>
</tr>
<tr>
<td>Hypertension (n (%))</td>
<td>139 (100)</td>
<td>115 (83)</td>
<td>64 (77)</td>
<td>51 (91)</td>
</tr>
<tr>
<td>Coronary artery disease (n (%))</td>
<td>139 (100)</td>
<td>18 (13)</td>
<td>8 (10)</td>
<td>10 (18)</td>
</tr>
<tr>
<td>CKD stage (n (%))</td>
<td>139 (100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td></td>
<td>5 (4)</td>
<td>5 (6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Grade 2</td>
<td></td>
<td>17 (12)</td>
<td>16 (19)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Grade 3a</td>
<td></td>
<td>22 (16)</td>
<td>19 (23)</td>
<td>3 (5)</td>
</tr>
<tr>
<td>Grade 3b</td>
<td></td>
<td>42 (30)</td>
<td>25 (30)</td>
<td>17 (30)</td>
</tr>
<tr>
<td>Grade 4</td>
<td></td>
<td>46 (33)</td>
<td>16 (19)</td>
<td>30 (54)</td>
</tr>
<tr>
<td>Grade 5</td>
<td></td>
<td>7 (5)</td>
<td>2 (2)</td>
<td>5 (9)</td>
</tr>
<tr>
<td>CKD aetiology (n (%))</td>
<td>139 (100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td>23 (17)</td>
<td>8 (10)</td>
<td>15 (27)</td>
</tr>
<tr>
<td>Hypertension</td>
<td></td>
<td>12 (9)</td>
<td>9 (11)</td>
<td>3 (5)</td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td></td>
<td>30 (22)</td>
<td>23 (28)</td>
<td>7 (13)</td>
</tr>
<tr>
<td>Congenital</td>
<td></td>
<td>8 (6)</td>
<td>7 (8)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Polycystic kidney disease</td>
<td></td>
<td>6 (4)</td>
<td>3 (4)</td>
<td>3 (5)</td>
</tr>
<tr>
<td>Obstructive</td>
<td></td>
<td>6 (4)</td>
<td>2 (2)</td>
<td>4 (7)</td>
</tr>
<tr>
<td>Interstitial</td>
<td></td>
<td>7 (5)</td>
<td>5 (6)</td>
<td>2 (4)</td>
</tr>
<tr>
<td>Other/unknown</td>
<td></td>
<td>47 (34)</td>
<td>26 (31)</td>
<td>21 (38)</td>
</tr>
<tr>
<td>Laboratory data</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum creatinine (mean±SD; mg/dL)</td>
<td>139 (100)</td>
<td>2.0±1.0</td>
<td>1.6±0.6</td>
<td>2.6±1.1</td>
</tr>
<tr>
<td>CKD-EPI eGFR (median [IQR]; mL/min/BSA)</td>
<td>139 (100)</td>
<td>33 [24 - 51]</td>
<td>43 [31 - 60]</td>
<td>26 [18 - 34]</td>
</tr>
<tr>
<td>uACR (median [IQR]; mg/g)</td>
<td>139 (42)</td>
<td>127 [22 - 479]</td>
<td>33 [18 - 144]</td>
<td>504 [92 - 1249]</td>
</tr>
<tr>
<td>uPCR (median [IQR]; mg/g)</td>
<td>139 (55)</td>
<td>327 [124 - 814]</td>
<td>301 [124 - 637]</td>
<td>345 [181 - 1381]</td>
</tr>
<tr>
<td>Merged uACR (median [IQR]; mg/g)</td>
<td>139 (81)</td>
<td>144 [22 - 532]</td>
<td>104 [19 - 269]</td>
<td>283 [83 - 993]</td>
</tr>
<tr>
<td>Haemoglobin (mean±SD; g/dL)</td>
<td>138 (99)</td>
<td>13.0±1.7</td>
<td>13.4±1.9</td>
<td>12.3±1.2</td>
</tr>
<tr>
<td><strong>KFRE estimates (median [IQR]; %)</strong></td>
<td>113 (81)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------------------------</td>
<td>----------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-year</td>
<td>1.4 [0.3 - 6.5]</td>
<td>0.6 [0.1 - 2.4]</td>
<td>6.4 [1.6 - 18.7]</td>
<td></td>
</tr>
<tr>
<td>5-year</td>
<td>5.4 [1.2 - 23.1]</td>
<td>2.1 [0.3 - 9.0]</td>
<td>22.5 [6.1 - 55.1]</td>
<td></td>
</tr>
</tbody>
</table>

**Multiplex biomarker values**

<table>
<thead>
<tr>
<th><strong>C-reactive protein (median [IQR]; ng/mL)</strong></th>
<th>139 (100)</th>
<th>3035 [1607 - 8244]</th>
<th>2610 [1416 - 5296]</th>
<th>4612 [2303 - 15181]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cystatin C (median [IQR]; ng/mL)</strong></td>
<td>139 (100)</td>
<td>3715 [2575 - 5000]</td>
<td>3241 [2115 - 4384]</td>
<td>4984 [3662 - 5739]</td>
</tr>
<tr>
<td><strong>C3a-desArg (median [IQR]; ng/mL)</strong></td>
<td>139 (100)</td>
<td>1762 [1010 - 2877]</td>
<td>2240 [1099 - 3392]</td>
<td>1467 [947 - 2178]</td>
</tr>
<tr>
<td><strong>D-dimer (median [IQR]; ng/mL)</strong></td>
<td>138 (99)</td>
<td>81 [41 - 153]</td>
<td>73 [34 - 143]</td>
<td>103 [49 - 172]</td>
</tr>
<tr>
<td><strong>EGF (median [IQR]; pg/mL)</strong></td>
<td>138 (99)</td>
<td>91 [54 - 126]</td>
<td>109 [58 - 134]</td>
<td>72 [47 - 104]</td>
</tr>
<tr>
<td><strong>FABP1 (median [IQR]; ng/mL)</strong></td>
<td>139 (100)</td>
<td>1.6 [0.7 - 3.0]</td>
<td>1.3 [0.6 - 2.7]</td>
<td>1.9 [1.2 - 3.8]</td>
</tr>
<tr>
<td><strong>Interleukin-8 (median [IQR]; pg/mL)</strong></td>
<td>139 (100)</td>
<td>3.7 [2.6 - 8.5]</td>
<td>3.9 [2.5 - 8.6]</td>
<td>3.6 [2.7 - 7.3]</td>
</tr>
<tr>
<td><strong>MIP-1-alpha (median [IQR]; pg/mL)</strong></td>
<td>130 (94)</td>
<td>4.8 [2.6 - 10.6]</td>
<td>3.9 [2.3 - 6.6]</td>
<td>7.5 [3.7 - 15.1]</td>
</tr>
<tr>
<td><strong>NGAL (median [IQR]; ng/mL)</strong></td>
<td>137 (99)</td>
<td>167 [116 - 253]</td>
<td>137 [104 - 194]</td>
<td>213 [173 - 327]</td>
</tr>
<tr>
<td><strong>sTNFR1 (median [IQR]; ng/mL)</strong></td>
<td>136 (98)</td>
<td>3.0 [2.1 - 4.6]</td>
<td>2.4 [1.9 - 3.2]</td>
<td>4.6 [3.5 - 6.8]</td>
</tr>
<tr>
<td><strong>sTNFR2 (median [IQR]; ng/mL)</strong></td>
<td>138 (99)</td>
<td>1.4 [0.8 - 2.2]</td>
<td>1.1 [0.6 - 1.6]</td>
<td>2.1 [1.3 - 2.5]</td>
</tr>
</tbody>
</table>

*BSA = body surface area; CKD-EPI = Chronic Kidney Disease-Epidemiology Collaboration; C3a-desArg = complement protein C3a (cleaved at C-terminal arginine); EGF = epidermal growth factor; eGFR = estimated glomerular filtration rate; FABP1 = fatty acid-binding protein-1; IQR = interquartile range; KFRE = kidney failure risk equation (4-variable; non-North America); MIP-1-alpha = macrophage inflammatory protein-1-alpha; NGAL = neutrophil gelatinase-associated lipocalin; SD = standard deviation; sTNFR1 = soluble tumour necrosis factor receptor-1; sTNFR2 = soluble tumour necrosis factor receptor-2; uACR = urine albumin-to-creatinine ratio; uPCR = urine protein-to-creatinine ratio.

Values are given as n (%) for categorical variables, or mean±SD for normally distributed continuous variables, unless otherwise indicated. Median [IQR] values are presented for continuous variables that are not normally distributed.

**Composite endpoint:** ≥40% decrease in CKD-EPI eGFR, doubling of serum creatinine, renal replacement therapy, or mortality.

**Merged uACR** represents a combination of measured uACR and calculated uACR from uPCR using the validated equation of Weaver et al. uACR values on the natural log scale were exponentiated such that presented values are in absolute units in mg/g.
Table 2. Duration of Follow-up and Incidence of Renal and Mortality Endpoints During the Study Period (n=139).\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Data available (n (%))</th>
<th>Total cohort (n=139)</th>
<th>Did not develop composite endpoint (n=83)</th>
<th>Developed composite endpoint (n=56)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of eGFR measurements (median [IQR])</td>
<td>139 (100)</td>
<td>22 [12 - 34]</td>
<td>19 [11 - 29]</td>
<td>27 [15 - 42]</td>
</tr>
<tr>
<td>Duration of renal functional follow-up (median [IQR]; years)\textsuperscript{c}</td>
<td>139 (100)</td>
<td>4.7 [3.5 - 5.2]</td>
<td>4.9 [4.1 – 5.2]</td>
<td>4.1 [1.8 – 5.2]</td>
</tr>
<tr>
<td>Duration of study follow-up (median [IQR]; years)\textsuperscript{d}</td>
<td>139 (100)</td>
<td>5.4 [4.7 - 5.7]</td>
<td>5.4 [4.8 – 5.7]</td>
<td>5.4 [4.4 – 5.7]</td>
</tr>
<tr>
<td>Slope of CKD-EPI eGFR (median [IQR]; mL/min/BSA/year)\textsuperscript{e}</td>
<td>129 (93)</td>
<td>-0.9 [-2.3 - 0.5]</td>
<td>0 [-1 – 1.6]</td>
<td>-2.2 [-3.9 - -1.4]</td>
</tr>
<tr>
<td>≥40% decrease in CKD-EPI eGFR (n (%))</td>
<td>139 (100)</td>
<td>38 (27)</td>
<td>0 (0)</td>
<td>38 (68)</td>
</tr>
<tr>
<td>Doubling of serum creatinine (n (%))</td>
<td>139 (100)</td>
<td>14 (10)</td>
<td>0 (0)</td>
<td>14 (25)</td>
</tr>
<tr>
<td>Required RRT (n (%))</td>
<td>139 (100)</td>
<td>21 (15)</td>
<td>0 (0)</td>
<td>21 (38)</td>
</tr>
<tr>
<td>Death from any cause (n (%))</td>
<td>139 (100)</td>
<td>15 (11)</td>
<td>0 (0)</td>
<td>15 (27)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} BSA = body surface area; CKD-EPI = Chronic Kidney Disease-Epidemiology Collaboration; eGFR = estimated glomerular filtration rate; IQR = interquartile range; RRT = renal replacement therapy; SD = standard deviation.

\textsuperscript{b} Composite endpoint: ≥40% decrease in CKD-EPI eGFR, doubling of serum creatinine, renal replacement therapy, or mortality.

\textsuperscript{c} Duration between date of study enrolment and date of final eGFR determination. eGFR values subsequent to RRT initiation were excluded.

\textsuperscript{d} Duration between date of study enrolment and date of last study follow-up or date of death.

\textsuperscript{e} Slope of CKD-EPI eGFR was calculated only for individuals with 3 or more eGFR values over at least 1 year.
Table 3. Random Forest Model Performance Metrics for Prediction of the Composite Renal and Mortality Endpoint (n=126).\(^{a,b,c}\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Predicted Probability Threshold &gt;10%</th>
<th>Predicted Probability Threshold &gt;30%</th>
<th>Predicted Probability Threshold &gt;50%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clinical</td>
<td>Biomarker</td>
<td>Clinical + Biomarker</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.92</td>
<td>0.96</td>
<td>0.98</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.36</td>
<td>0.24</td>
<td>0.21</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>0.47</td>
<td>0.44</td>
<td>0.43</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>0.87</td>
<td>0.90</td>
<td>0.94</td>
</tr>
</tbody>
</table>

\(^{a}\)Three types of random forest classification models were implemented to evaluate prediction of the composite renal and mortality endpoint: clinical variables alone (age, gender, hypertension, diabetes, and baseline eGFR), serum biomarkers alone, and clinical variables plus serum biomarkers.

\(^{b}\)A leave-one-out cross-validation approach was implemented, which consisted of excluding one individual from training the random forest model. Subsequently, the trained random forest model predicted the class of the individual excluded during model training. This process was repeated iteratively for each individual in the dataset such that a predicted class was assigned to each study participant by each of the three random forest model types.

\(^{c}\)Model performance metrics were calculated across three probability thresholds (10%, 30%, and 50%) for labelling patients as having developed the composite renal and mortality endpoint. For example, an individual with a predicted probability of the composite renal and mortality endpoint by a random forest model of 45% would be labelled as having developed the composite renal and mortality endpoint by the first two thresholds evaluated, but not the latter.
Figure Legends

Figure 1. Univariate associations between serum biomarkers and a composite renal and mortality endpoint.

A-B: Variables which exhibited a significant inverse relationship with the composite renal and mortality endpoint, including epidermal growth factor (A) and C3a-desArg (B).

C-D: Biomarkers with a modest positive relationship with the composite renal and mortality endpoint, including MIP-1-alpha (C) and C-reactive protein (D).

E-H: Biomarkers which displayed a strong positive relationship with the composite renal and mortality endpoint, including sTNFR1 (E), sTNFR2 (F), NGAL (G), and cystatin C (H).

Individuals who developed the composite renal and mortality endpoint are identified as 100% on the y-axis and as triangular points. Individuals who did not develop the composite renal and mortality endpoint are identified as 0% on the y-axis and as circular points.

Biomarkers which displayed an inverse association with the composite renal and mortality endpoint are coloured in blue, those with a strong positive association are coloured in red, and those with modest positive associations are presented in a shade intermediate between both colours.

Log-transformation of serum biomarkers was performed prior to modelling.

Odds ratios from logistic regression models are expressed per one unit change in natural logarithm biomarker concentrations.

95% confidence interval is represented by navy shading surrounding pink regression curve.

The size of individual data points is scaled by baseline eGFR to illustrate relationships between serum biomarkers and kidney function at enrolment. Larger points = higher eGFR; smaller points = lower eGFR.

Composite renal and mortality endpoint: ≥40% decrease in CKD-EPI eGFR, doubling of serum creatinine, renal replacement therapy, or mortality.

Figure 2. Principal components analysis illustrates relationships between biomarker expression, CKD stage, and a composite renal and mortality endpoint.

A: Unsupervised clustering of patients by principal components analysis identifies global shifts in biomarkers across categorical CKD stages, grouped as grades 1 and 2 (eGFR ≥60 mL/min/BSA), grade 3 (eGFR 30-59 mL/min/BSA), and grades 4 and 5 (eGFR <30 mL/min/BSA).

B: Unsupervised clustering of patients by principal components analysis identifies global shifts in biomarker expression profiles between those who did and did not develop a composite renal and mortality endpoint during follow-up.
C: A loadings plot from principal components analysis reveals the influential biomarkers which drive the shifts in biomarker expression across CKD stages and when stratified by the composite renal and mortality endpoint. Individuals with advanced CKD who developed the composite renal and mortality endpoint had higher expression of biomarkers in the right of the plot, including sTNFR1, sTNFR2, NGAL, and cystatin C. Individuals with earlier stage CKD who did not develop the composite renal and mortality endpoint had higher expression of protective factors in the upper left corner of the plot including C3a-desArg and epidermal growth factor.

The colour of the points illustrates the directionality of the relationships between biomarkers and the composite renal and mortality endpoint (blue = inverse, red = strong positive, while modest positive relationships are coloured in between both). The shape of the points represents statistical significance of the relationship between biomarkers and the composite renal and mortality endpoint by univariate logistic regression (circle = statistically significant, triangle = not statistically significant).

In panels A, B, and C, the x- and y-axes represent principal components 1 and 2, respectively.

Composite renal and mortality endpoint: ≥40% decrease in CKD-EPI eGFR, doubling of serum creatinine, renal replacement therapy, or mortality.

Figure 3. A supervised machine learning approach (random forest classifier) illustrates the added predictive value of multiple serum biomarkers for a composite renal and mortality endpoint, both when considered alone and in addition to conventional clinical variables.

A: Decision tree classification of the composite renal and mortality endpoint by serum biomarkers in the study cohort. The decision tree highlights the predictive value of simultaneously assessing multiple serum biomarkers. In this decision tree, the 3 biomarkers are ranked by their proximate level of importance to correct classification of the composite renal and mortality endpoint, from sTNFR1 (highest) to C3a-desArg (lowest).

Individuals with low sTNFR1 values (<3 ng/mL) had a relatively low risk of the composite renal and mortality endpoint (12%). However, not all individuals with high sTNFR1 values had the same risk of the composite renal and mortality endpoint. Those with high sTNFR1 (≥3 ng/mL) coupled with low NGAL values (<156 ng/mL) had a 39% risk of the composite renal and mortality endpoint, while those with high sTNFR1 (≥3 ng/mL), high NGAL (≥156 ng/mL), but also high C3a-desArg values (≥2,368 ng/mL) had a 44% risk of the composite renal and mortality endpoint. Conversely, individuals with the triad of high sTNFR1 (≥3 ng/mL), high NGAL (≥156 ng/mL), and low C3a-desArg (<2,368 ng/mL), which accounted for approximately 20% of the study cohort, almost universally (96%) developed the composite renal and mortality endpoint during follow-up.
Biomarker values in the decision tree are coloured in a continuous gradient from left to right from blue (lower risk) to red (higher risk).

B: Receiver operating characteristic (ROC) curves for 3 types of random forest classification models of the composite renal and mortality endpoint: clinical variables alone (age, gender, hypertension, diabetes, and baseline eGFR) (green), serum biomarkers alone (orange), and clinical variables plus serum biomarkers (purple). A leave-one-out cross-validation approach was implemented for the random forest models. The plot illustrates incremental improvements in correct prediction of the composite renal and mortality endpoint across the 3 model types.

AUC values and associated 95% confidence intervals for the 3 model types are presented in the inset table: 0.78 for clinical variables alone, 0.81 for serum biomarkers alone, and 0.83 for clinical variables plus serum biomarkers.

C-E: Dotplots of variable importance across the 3 random forest classification models of the composite renal and mortality endpoint: clinical variables alone (age, gender, hypertension, diabetes, and baseline eGFR) (C), serum biomarkers alone (D), and clinical variables plus biomarkers (E). eGFR was the most important clinical variable, while sTNFR1 and NGAL were the biomarkers which provided the most predictive value to the models.

All 5 clinical variables are presented (C), while the top 8 most important variables are presented for the biomarkers alone and clinical variables plus biomarker models (D and E).

The dots are coloured in a continuous gradient from navy (lower variable importance) to yellow (higher variable importance).

Composite renal and mortality endpoint: ≥40% decrease in CKD-EPI eGFR, doubling of serum creatinine, renal replacement therapy, or mortality.

Figure 4. C-reactive protein, NGAL and C3a-desArg independently predict time to development of a composite renal and mortality endpoint (Cox proportional hazards regression).

A: Forest plot of parsimonious Cox proportional hazards regression model incorporating clinical variables and serum biomarkers predictive of a composite renal and mortality endpoint (n=126).

C-reactive protein (adjusted hazard ratio [aHR], 1.4; 95% confidence interval [95% CI], 1.1 to 1.9) and NGAL (aHR, 2.7; 95% CI, 1.3 to 5.8) were positively associated with time to the composite renal and mortality endpoint. C3a-desArg values were inversely associated with time to the composite renal and mortality endpoint (aHR, 0.5; 95% CI, 0.3 to 0.9).

B-D: Plots of survival without the composite renal and mortality endpoint derived from the fully adjusted, multivariate clinical plus biomarker Cox proportional hazards regression
model presented in the Forest plot in panel A, stratified by tertiles of serum C-reactive protein (B), NGAL (C) and C3a-desArg (D).

Biomarker values were log-transformed for modelling.

Hazard ratios from Cox models are expressed per one unit change in natural logarithm biomarker concentrations.

Composite renal and mortality endpoint: ≥40% decrease in CKD-EPI eGFR, doubling of serum creatinine, renal replacement therapy, or mortality.
Figure 1

Composite Endpoint • No ▲ Yes eGFR ▲ 10 ▶ 10

Inverse associations

Epidermal growth factor (pg/mL)

C3a–desArg (ng/mL)

MIP–1–alpha (pg/mL)

C–reactive protein (ng/mL)

Modest positive associations

Strong positive associations

sTNFR1 (ng/mL)

sTNFR2 (ng/mL)

NGAL (ng/mL)

Cystatin C (ng/mL)
Figure 2

(A) Scatter plot showing the relationship between PC1 and PC2 with different CKD stages. CKD Stage: G1/2 (blue), G3 (orange), G4/5 (green).

(B) Scatter plot for Composite Endpoint: No (red) and Yes (blue, inverse relationship).

(C) Scatter plot showing the relationship to Composite Endpoint with markers for statistical significance by Univariate Logistic Regression: Not significant (up arrow), Significant (down arrow), Inverse, Modest positive, Strong positive.
sTNFR1 (ng/mL); n = 126
NGAL (ng/mL); n = 61
C3a–desArg (ng/mL); n = 43

Composite Endpoint

<table>
<thead>
<tr>
<th>Variable Importance (Mean Decrease in Accuracy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical Variables Alone</td>
</tr>
<tr>
<td>Biomarkers Alone</td>
</tr>
<tr>
<td>Clinical Variables + Biomarkers</td>
</tr>
</tbody>
</table>

Figure 3
### Table 1: Risk Factors for Composite Endpoint

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Reference</th>
<th>Hazard Ratio</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (N=126)</td>
<td>0.97 (0.96 - 1.00)</td>
<td>0.025 *</td>
<td></td>
</tr>
<tr>
<td>Gender (N=96)</td>
<td>Female</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Hypertension (N=70)</td>
<td>Yes</td>
<td>3.34 (1.61 - 6.94)</td>
<td>0.001 **</td>
</tr>
<tr>
<td>Diabetes (N=30)</td>
<td>Yes</td>
<td>1.36 (0.48 - 3.87)</td>
<td>0.566</td>
</tr>
<tr>
<td>eGFR (N=126)</td>
<td>0.96 (0.93 - 0.99)</td>
<td>0.003 **</td>
<td></td>
</tr>
<tr>
<td>C-reactive protein (N=126)</td>
<td>1.40 (1.07 - 1.84)</td>
<td>0.015 *</td>
<td></td>
</tr>
<tr>
<td>NGAL (N=126)</td>
<td>2.77 (1.25 - 6.13)</td>
<td>0.012 *</td>
<td></td>
</tr>
<tr>
<td>C3a–desArg (N=126)</td>
<td>0.58 (0.36 - 0.96)</td>
<td>0.034 *</td>
<td></td>
</tr>
</tbody>
</table>

# Events: 48; Global p–value (Log–Rank): 1.1677e–13
AIC: 375.86; Concordance Index: 0.83

### Figure 4

#### B: C-reactive protein

#### C: NGAL

#### D: C3a–desArg

Tertiles:
- T1
- T2
- T3

Survival without composite endpoint (%) vs. Time (years)